Prenatal microRNA miR-200b therapy improves nitrofen-induced pulmonary hypoplasia associated with congenital diaphragmatic hernia.

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Online Supplementary Data

Methods

Cell culture

BEAS-2B cells (human bronchial epithelial cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in coated tissue culture dishes with bronchial epithelial cell growth medium (BEGM, Lonza, CA). Cultures were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2. Cells were detached from dishes using 0.25% Trypsin - 0.53mM EDTA solution containing 0.5% polyvinylpyrrolidone (PVP) during passaging.

RNA extraction and RT-qPCR

Lungs from Embryonic day (E) 13, 15, 18 and 21 pups were isolated in ice cold PBS, snap-frozen in liquid nitrogen and stored at -80°C until processed. BEAS-2B cells were scraped after the experiment and snap frozen in liquid nitrogen and stored at -80°C until processed. Total RNA from embryonic lung tissues or BEAS-2B cells was extracted using the miRCURY™ Isolation Kit (Ambion), per the manufacturer's instructions. For microRNA analysis, cDNA was randomly primed from 20 ng total RNA using the Exiqon cDNA synthesis kit. RT-qPCR was

subsequently performed using locked nucleotide acid (LNA) primers for rno-miR-200b, U6 and miR-103 (as endogeneous controls). All primer sequences are provided in Supplementary Table 1. We used the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon) in a total reaction volume of 20µl. Briefly, RT-qPCR was performed in triplicate with 1:80 dilution of cDNA using the SYBR green PCR system on a ABI 7500 Real-Time PCR machine (Applied Biosciences). Data were collected and analyzed using ABI 7500 v1.4.0 software (Applied Biosciences). MicroRNA expression levels were determined using the relative quantification method of the ABI 7500 v1.4.0 software.

Rno-LNA-miR-200b was hybridized with double-digoxigenin-labeled LNA probes (Exiqon, supplementary Table 2) for 1 hour at 52°C (30 °C below the calculated RNA melting temperature). The probes were detected with an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (1:500) (Roche, Mannheim, Germany). Sections were immunostained with 1-step NBT/BCIP solution containing 1 mM levamisole (Thermo Scientific, Rockford, IL, USA). The slides were then counterstained with methylgreen (Sigma-Aldrich, St. Louis, MO, USA). Digital microscopy was performed using the ScanScope CS system (Aperio, Vista, CA, USA).

Loss- and gain-of-function study and SMAD luciferase activity assays

To identify the best primary lung cell line to perform loss- and gain-of-function studies, we examined the expression of miR-200b in several lung cell lines (data not shown). We observed the highest miR-200b expression in BEAS-2B cells. We treated these cells with 10µM of nitrofen. After one hour of incubation, treated or untreated cells were co-transfected with 0.5 µg/ml of the SMAD reporter construct (Cignal SMAD Reporter kit, SABiosciences, Frederick,

MD, USA) and 0.01 µg/ml of LNA-hsa-miR-200b inhibitor, mimic or negative control (Exiqon)—using the X-tremeGENE siRNA Transfection Reagent (Roche). SMAD luciferase activity was measured as previously described (supplementary data) [11]. Briefly, after 48h, the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Treated or untreated cells were lysed using 100 µl of passive lysis buffer of the kit and put on a shaker for 15 min, and the supernatant of the lysates were collected after centrifugation. 20µl of the supernatants was used for luciferase activity measurement using a (core BMG POLARstar OPTIMA) Microplate Reader. Luciferase activity between the treated groups was compared after normalization with Renilla luciferase activity.

Wester blotting

Specific proteins were detected with the following antibodies: anti-SMAD2/3 (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-SMAD2 (Cell Signaling Technology), anti-ZEB1 and anti-ZEB2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TGF-β2 antibody (1:200, Abcam, Cambridge, MA, USA) anti-GAPDH (1:6000; Abcam, MA, USA), HRP-conjugated goat–anti-rabbit secondary antibody (Bio-Rad) and HRP-conjugated goat–anti-mouse secondary antibody (Bio-Rad). Exposed films were scanned and densitometry was performed on unmodified output images after background subtraction using ImageJ software (Wayne Rasband, NIH, USA) in a blinded fashion. Densitometric values were normalized against the corresponding GAPDH values.

Statistical analysis

All data are presented as mean +/- standard error of mean, from a minimum of three independent experiments. Statistical significance was determined by one-way ANOVA, *student's t*-test or Chi square as indicated in the figure legends. A p-value ≤0.05 was considered significant.

Results:

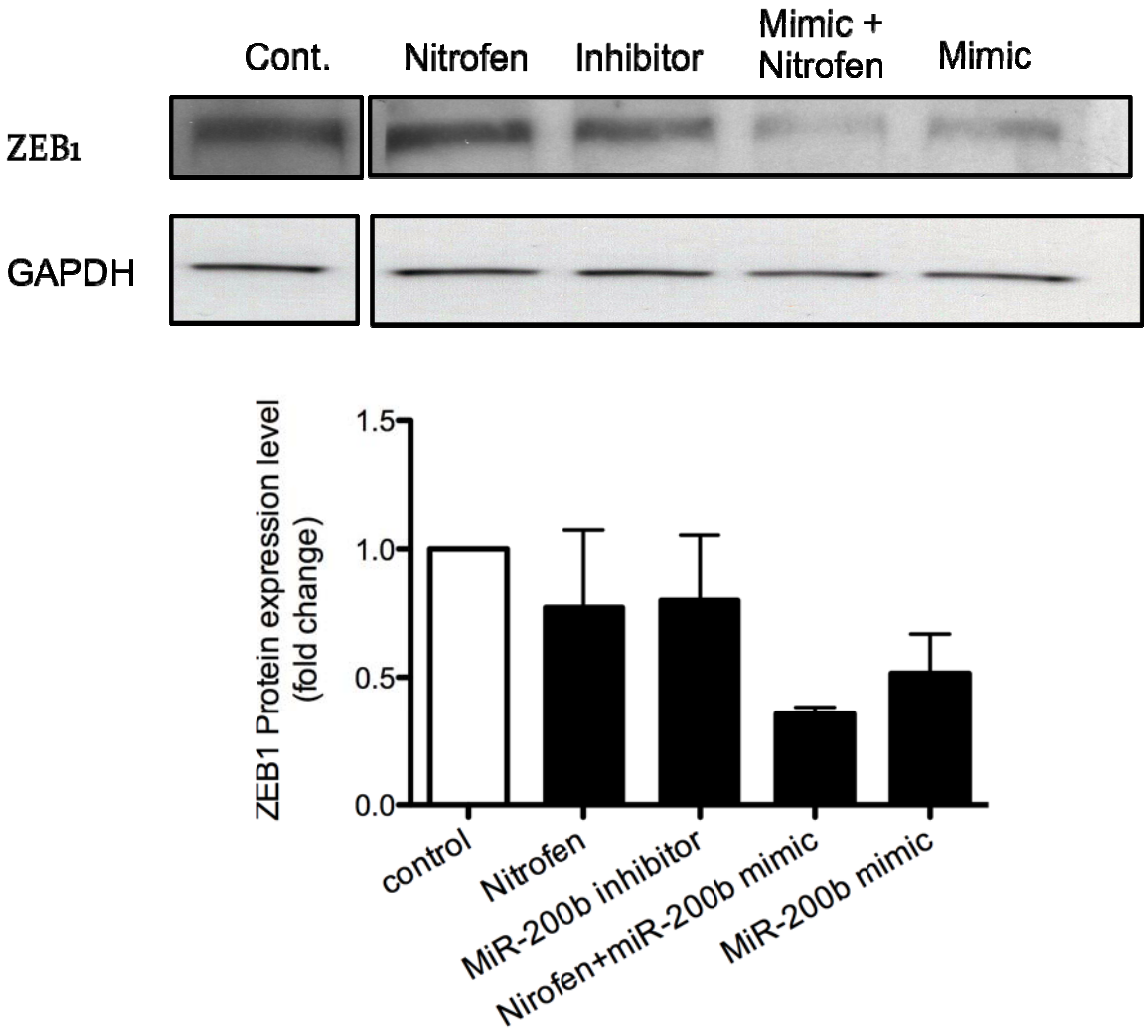


Figure S1. Inhibition of miR-200b or nitrofen does not promote ZEB1 expression. Western blotting with ZEB1 antibody on BEAS-2B cells treated with nitrofen, miR-200b inhibitors, miR-200b mimics and a combination of miR-200b and nitrofen did not show any differences in ZEB1 protein abundance (representative image of three independent experiments).

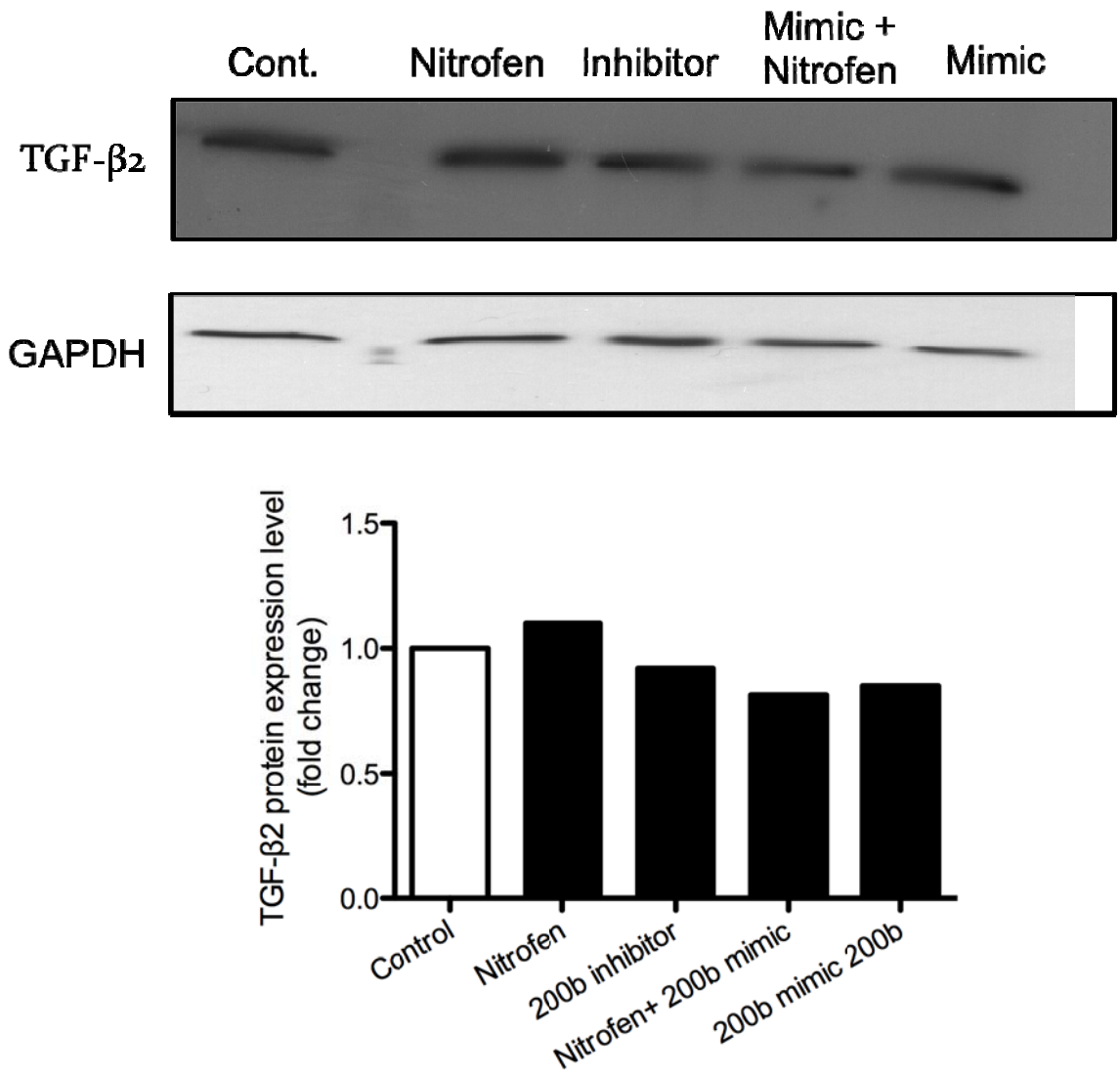
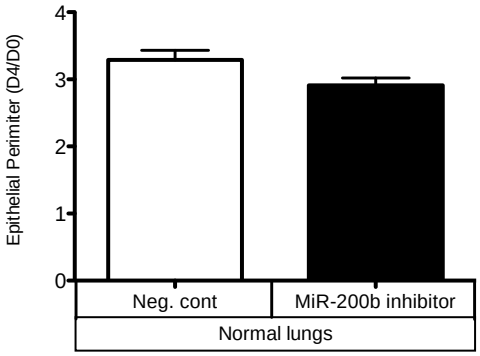


Figure S2. MiR-200b does not influence TGF-β2 expression in BEAS-2B cells. Western blotting with TGF-β2 antibody on BEAS-2B cells treated with nitrofen, miR-200b inhibitors, miR-200b mimics and a combination of miR-200b and nitrofen did not show any differences in TGF-β2 protein (representative image of two independent experiments).

a



b

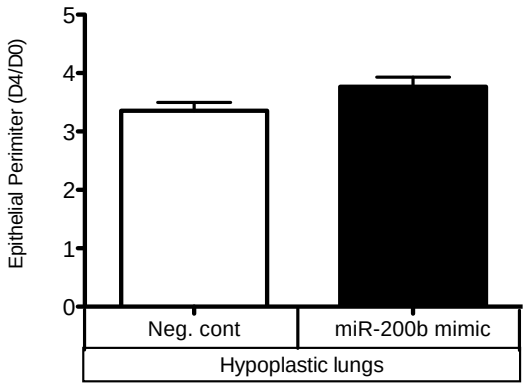


Figure S3. miR-200b had no significant effect on lung epithelial perimeter in normal or hypoplastic lung during *ex-vivo* culture. (a) Normal lung explants treated once with miR -200b inhibitor, after 4 days in culture, Epithelial perimeter was measured. (b) hypoplastic lung explants treated once with miR-200b mimic, after 4 days in culture, Epithelial perimeter was measured.

a b

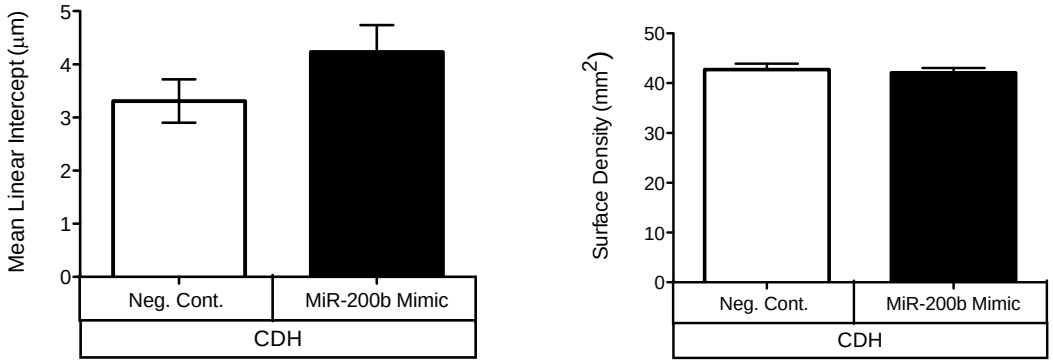


Figure S4. The mean linear intercept of lungs with CDH in miR-200b group were higher but the differences were not significant. Pregnant rats were injected intravenously with miR-200b or oligo negative control of pregnant rat at E9 after nitrofen gavaging.

Movie S1. Prenatal miR-200b therapy improves ventilation in nitrofen-treated newborn rats. Following nitrofen gavaging on E9, pregnant rats were injected with 5 mg/kg miR-200b mimics or negative control oligonucleotides. Nitrofen-treated newborn rats in the miR-200b group displayed normal pink skin color and normal work of breathing as opposed to the nitrofen-treated newborns in the negative control group, who displayed increased work of breathing and a dark purple skin color, suggesting disturbed ventilation.

Outcome / Treatment	Pups with CDH	Pups without CDH	Total
miR-200b	32	89	121
Neg. Cont.	36	24	60

Table S1: CDH incidence after prenatal transplacental miR-200b therapy. Number of pups with or without CDH (non-CDH) in the two treatment groups (the data is represented from at least three independent experiments)

LNA microRNA primer set	Description
U6 snRNA (hsa, rno,mmu)	endogenous control
rno-miR-200b	tested microRNA

Table S2: Locked nucleic acid (LNA) primer sets used in real-time quantitative PCR (RT-qPCR)

Probe	Sequence	Concentration	Hybridization Temperature
rno-miR-200b rat	GTCATCATTACCAGGCAGTATTA	100 nM	51°C
scramble-miR negative control	GTGTAACACGTCTATACGCCCA	100 nM	57°C
U6, hsammurno positive control	CACGAATTTGCGTGTCATCCTT	0.1 nM	54°C

Table S3: double DIG labeled miRCURY LNA™ miRNA detection probes used for in situ hybridization (Exiqon).